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PrfA activation at the single cell level in *Listeria monocytogenes*

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Abstract

The human pathogen *Listeria monocytogenes* is able to transition from environmental saprophyte to facultative intracellular bacteria. In this process, virulence gene expression is controlled by the positive regulatory factor A (PrfA). Recent studies at the single cell level have shown that gene expression in response to stress exposure is stochastic in individual bacteria cells. Current studies applied those general findings to *Listeria* cells, revealing that PrfA as well is not regulated consistently, but that PrfA activity differs between individual cells. The aim of this study was to elucidate the mechanism by which *Listeria* regulates PrfA activation at the single cell level and whether this property is heritable or not. A reporter fusion, namely an eGFP sequence, integrated following the PrfA dependent promoter (P*hly*), was used to visualize the activation after heat stress exposure. Fluorescence activated cell sorting (FACS) was used to distinguish PrfA positive and negative cells. After passaging and sorting PrfA activating versus non-activating cells over several generations, two stable fluorescent phenotypes emerged. A comparison between the genome of the PrfA positive and its parent strain revealed a single-nucleotide polymorphism (SNP) in the CDS of LMRG_02823, as well as a mutation in the 5'UTR of LMRG_00195, both LPXTG family cell wall associated proteins regulated via small RNAs. The link between those mutations and PrfA activation is currently being investigated.

Keywords: *Listeria monocytogenes*, PrfA, single cell, stochastic gene regulation, FACS

Zusammenfassung

Listeria monocytogenes ist einerseits ein saprophytäres Umweltbakterium, kann aber auch als fakultativ intrazelluläres Pathogen agieren. Die Virulenzgenexpression von *L. monocytogenes* wird hauptsächlich über den Mastertranskriptionsfaktor PrfA reguliert. Forschungsarbeiten der letzten zehn Jahre haben gezeigt, dass die Genexpression auf Einzelzellebene nicht wie erwartet immer nach dem gleichen Schema abläuft, sondern bei den individuellen Zellen je nach Stressreiz variiert. Eine aktuelle Studie hat diese Erkenntnisse auf *L. monocytogenes* angewandt, mit dem Ziel, die Aktivierung von PrfA durch die einzelne Bakterienzelle zu untersuchen. Und auch hier scheinen die Zellen eine gewisse Autonomie zu besitzen. Ziel dieser Arbeit war es herauszufinden, wie und weshalb eine einzelne Listerienzelle den Mastertranskriptionsfaktor PrfA aktiviert und inwiefern diese Aktivierungseigenschaft "vererbt" ist. Mittels einer Reporter Fusion, genauer eGFP gekoppelt an den PrfA abhängigen Promoter des *hly* Gens (P*hly*), wurde die stressinduzierte PrfA Aktivierung sichtbar gemacht. Mithilfe der Fluoreszenz-Durchflusszytometrie (FACS) wurde die Zellpopulation in PrfA positiv und negativ unterteilt. Durch weiteres Passagieren dieser Subpopulationen entstanden zwei klare Phänotypen: solche die PrfA stärker aktieren und solche mit einer tieferen Aktivierungsrate. Mittels PacBio Sequencing wurde versucht den Ursprung dieser Phänotypen auszumachen. Wir fanden im PrfA+ Stamm (ILS G3-0005) PrfA in seiner Wildtyp-Form vor, was die Hypothese der Heritabilität bestätigt. Des Weiteren wurde einerseits ein Einzelnukleotid-Polymorphismus in der 5'UTR von Lmo1799 identifiziert, welche die Zielsequenz für eine sRNA, genannt Rli27, darstellt. Die zweite Mutation befindet sich in der 5'UTR von Lmo0514, einem Internalin der LPXTG Familie. Wir konnten jedoch bis anhin keine Verbindung zum PrfA positiven Phänotyp des Stammes ILS G3-0005 herstellen.

Introduction

Listeria monocytogenes

The gram-positive bacterium *Listeria monocytogenes* (*L. monocytogenes*) is responsible for bacterial infections in humans as well as animals (Murray 1955) and is therefore an important zoonosis, especially in ruminants (Pearson et al. 1990). Transmission is mainly foodborne and causes infrequent illness in healthy humans resulting in self-limiting febrile gastroenteritis (Pearson et al. 1990; Wing et al. 2002; Ooi et al. 2005). However, in rare cases, but especially in the YOPI group, including young (mainly neonates), old, pregnant and immunodeficient people, it may cause life-threatening bacteremia, (meningo-) encephalitis, and abortions in pregnant individuals (Smerdon et al. 2001; Guevara et al. 2009). Even though Listeriosis in humans is a rather rare disease, it poses a serious public health and food safety issue due to the high mortality rate of up to over 20% associated with central nervous system infections by *L. monocytogenes* (Guevara et al. 2009).

L. monocytogenes is a small, facultatively anaerobic bacterium which possesses five to six peritrichous flagella which render it motile at room temperature (20°C). The flagella are downregulated at 37°C (Peel et al. 1988). Even though optimal growth occurs at 37°C, *L. monocytogenes* is psychrotolerant and can grow well at refrigerator temperature (4-10°C). In addition it also tolerates a wide range of pH and large differences in osmolarity (Ryan et al. 2008; Mataragas et al. 2014). Those abilities enable the transition of *L. monocytogenes* from ubiquitous, free living and motile saprophyte in soil and decaying vegetation, to an intracellular pathogen, able to cause severe infections. A third lifestyle as biofilm producer was described by Lemon et al. (Lemon et al. 2010).

The Positive regulatory factor A (PrfA)

The capacity of *L. monocytogenes* to switch its lifestyle drastically, requires a specialized regulation system for the expression of virulence genes (Mengaud et al. 1991; Freitag et al. 1993; Freitag et al. 1994; Freitag et al. 2009). It is featured by the transcriptional activator PrfA (positive regulatory factor A), which was first described by Leimeister-Wächter et al. (1990) as a regulatory factor for the transcription of the *hly* gene. Further studies have proven its regulatory influence not only on the *hly* gene, but on up to 70 genes, whereas 10 genes are part of the core regulon of PrfA (Milohanic et al. 2003). Those 10 core virulence genes are sufficient to promote intracellular survival and the spread from cell to cell of *L. monocytogenes*. PrfA is therefore referred to as master virulence regulator (de las Heras et al. 2011).

PrfA itself is controlled on three different levels. Transcriptional regulation occurs via three promoters: two promoters, P*prfa*1 and P*prfa*2 are located immediately upstream of *prfA* and generate initial levels of PrfA protein. A third, bicistronic transcript originates from read-through off the promoter of *plcA* upstream of *prfA*. This third promoter is PrfA-dependent and thus results in an autoregulatory feedback loop. Post-transcriptional regulation of PrfA is mediated by the formation of secondary structures in the mRNA. A stem-loop structure that forms at temperatures below 30°C includes the ribosomal binding site and prevents translation. At 37°C, this structure is de-stabilized and translation occurs. Thirdly, there is the posttranslational regulation of PrfA activity which is still little-known about. PrfA exists in a weakly active state until it binds to an unknown cofactor or is somehow modified. We know that activated PrfA protein is required for optimal expression of virulence genes (Goldfine et al. 2007), however the actual signal and molecular mechanism by which PrfA itself is activated is yet to be found. Thus, a lot of work has been done the past years concerning this matter, revealing a complex regulation system suggesting different signal pathways as well as functional changes of PrfA.

Since there are constitutively active PrfA* mutations, consisting in single amino acid substitutions, it would make sense that the activation is due to conformational changes (Freitag et al. 2009; Lobel et al. 2015). Furthermore, PrfA activation mechanisms via metabolic signals are known, for example the regulation by sugar availability. PrfA activity is low in the presence of sugars transported by the PEP-dependent PTS system, for example glucose and cellobiose. On the other hand, non-PTS host-derived sugars such as glucose-1-phosphate (=phosphorlyated hexose) and glycerol initiate the transcription of *PrfA* and PrfA activity (Mertins et al. 2007; Joseph et al. 2008; Milenbachs et al. 1997; Lobel et al. 2015). Previous studies also found a positive effect of activated charcoal on PrfA activation (Ermolaeva et al. 2004; Ripio et al. 1996; Ripio et al. 1997). Ermolaeva et al. (2004) displayed that the 'charcoal effect' is due to the adsorption of a diffusible autorepressor agent secreted by *L. monocytogenes* during exponential growth. Furthermore, PrfA is known to be expressed by *L. monocytogenes* when cultivated in BHI media but remains inactive (Renzoni et al. 1997; Lobel et al. 2015), which raises further questions not only about possible cofactors or conformational changes but about the localization of the activation signal and the role of the host. Freitag and McGann found *L. monocytogenes* to be highly active when entering the host cell at 37°C (Freitag, Rong, and Portnoy 1993; McGann, Wiedmann, and Boor 2007). Those findings support the existence of a putative host signal. Reniere et al. (2015) confirmed this hypothesis by discovering that full transcriptional activation of PrfA needs allosteric binding to host glutathione. Those results are supported by a mutant in glutathione synthase, resulting in reduced virulence gene expression (Reniere et al. 2015; Reniere et al. 2016). A recent study found, that exposure to 45° C leads to the activation of PrfA at the single cell level (Guldimann et al. submitted).

Despite these results, we are still uncertain of the PrfA activation mechanisms. A better understanding might allow us to inhibit the invading process of *L. monocytogenes* during an infection and thus facilitate preventive measures.

Gene expression at the single cell level

Virulence gene expression in *L. monocytogenes* is governed by a complex regulatory network that involves, among other regulatory factors, PrfA as a main transcriptional regulator. Regarding these complex efforts, one would expect the cells to act similarly at population level. However, different studies showed that cells of a clonal population exposed to environmental changes react differently at the single cell level, meaning that some cells express stress relevant genes and some do not (Elowitz et al. 2002; Rosenfeld et al. 2005; Bintu et al. 2016). Guldimann et al. (submitted) applied those general findings to the *Listeria* gene expression system and found that exposure of *L. monocytogenes* to 45°C heat stress lead to an activation of PrfA in around 50% of all cells, showing that PrfA activation as well is regulated differentially at the single cell level. These results raised further questions - these included: what is the molecular mechanism behind PrfA activation at the single cell level? Does PrfA activation confer an advantage to an individual cell? Does the whole population profit from PrfA activation in a sub-population of cells? Finally, is this activation property passed on to the next generation?

The discovery of fluorescent proteins in combination with the development of high-throughput systems like flow cytometry enabled researchers to start analyzing the gene expression at the single-cell level (Chalfie et al. 1994; Shapiro 2003). Fusions between the sequence of the fluorescence protein and the target gene, enabled the detection of gene expression at the single cell level.

The fluorescent reporter strain for PrfA activity (Guldimann et al. submitted) was already available in the lab. The construct consists of a eGFP coding sequence codon-optimized for *L. monocytogenes* with a PrfA-dependent promotor (P*hly*) to detect PrfA activity. *hly* was chosen because of its exclusive regulation by PrfA. The strain was built by using pPL2, a site-specific integration vector for *L. monocytogenes*.

Research questions and hypothesis

The aim of this project was to address the following questions. What are the factors that lead an individual cell to activate PrfA while others in the same clonal population do not? Is the fact that an individual cell activates PrfA an inherent property of that cell, or is PrfA activation a stochastic process that happens arbitrarily? We hypothesized that the activation pattern is a heritable property of a cell, meaning that we have a PrfA active vs PrfA non-active population and is not stochastic.

Materials and Methods

Bacterial strains and growth conditions

The *L. monocytogenes* and *L. innocua* strains used in this study are listed in Table 1. To detect active PrfA, we used *L. monocytogenes* 10403s carrying a fluorescent reporter fusion consisting of an eGFP coding sequence fused to a PrfA dependent promoter (P*hly*::eGFP), genomically integrated in single copy (FSL G3-0073) (Guldimann et al. submitted). All strains were kept as frozen stocks in BHI + 15% glycerol at -80°C. For each experiment, *Listeria* strains were freshly streaked from frozen stocks on a BHI plate (Oxoid, Hampshire, UK) and incubated for 18h at 37°C. Overnight cultures were obtained by inoculating a single colony into 5ml of Brain Heart Infusion broth (BHI; Oxoid, Hampshire, UK) and incubated for 18h at 37°C with shaking at 200 rpm. These overnight cultures were were diluted 1:100 into 5ml fresh BHI and grown to an OD590 of 0.4 and incubated at 37°C with shaking at 200 rpm to obtain mid-log phase cultures.

Table 1: Bacterial strains

Bacterial strains					
Designation	Species	Name	Relevant Genotype	assignment	Reference
FSL G3-0007	*Listeria monocytogenes*	10403s	wt	wt Invasion assay; negative control FACS	Bishop and Hinrichs 1987
FSL G3-0073	*Listeria monocytogenes*	10403s	P*hly* ::*eGFP*	reporter	Guldimann et al., submitted
FSL G3-0099	*Listeria monocytogenes*	10403s	constitutive GFP	positive control FACS	Balestrino 2010
JF 5051	*Listeria innocua*		wt	negative control Invasion assay	gift from Joachim Frey, Institute for Veterinary Bacteriology, University of Bern
B2-0046	*Listeria monocytogenes*	10403s	*ΔPrfA*	negative control acid & hemolysis assay	Wong and Freitag 2004
B2-0237	*Listeria monocytogenes*	10403s	*PrfA** G145	positive control acid & hemolysis assay	Bergholz 2009
p6+	*Listeria monocytogenes*	10403s	FSL G3-0073 derivative	6th passage of G3-0073	this study
p6-	*Listeria monocytogenes*	10403s	FSL G3-0073 derivative	6th passage of G3-0073	this study
ILS G3-0005	*Listeria monocytogenes*	10403s	FSL G3-0073 derivative	6th passage - colony 6 PrfA+	this study
ILS G3-0006	*Listeria monocytogenes*	10403s	FSL G3-0073 derivative	6th passage - colony 6 PrfA-	this study
ILS G3-0007	*Listeria monocytogenes*	10403s	FSL G3-0073 derivative	6th passage - colony 3 PrfA+	this study
ILS G3-0008	*Listeria monocytogenes*	10403s	FSL G3-0073 derivative	6th passage - colony 3 PrfA-	this study

Fluorescence-activated cell sorting

To activate PrfA, we used the protocol by Guldimann et al. (submitted) that has been shown to induce PrfA activity in about 50% of individual cells in a *L. monocytogenes* population. In short, log-phase cultures of FSL G3-0073 and the respective control strains FSL G3-0099 and 10403s were diluted 1:20 into phosphate-buffered saline (PBS, Gibco Thermo Fisher, Reinach, Switzerland) preheated to 45°C and incubated at 45°C with shaking at 200rpm for 60min. To lower the background generated from the PBS in the FACS analysis, sterilised PBS was bought and a new aliquot was used for each experiment. Apart from the samples containing bacteria, the following controls were used in the FACS experiments (i) pure PBS; (ii) to test the background generated by the BHI medium still present in the samples, the cell-free supernatant of the negative control was diluted in PBS 1:20 and incubated at 45°C for 60 min (BHI control); (iii) a third control was established by exposing the wildtype (wt) strain to the same procedures as the reporter strain, namely the inducing step and the time period needed for the FACS (procedure control).

The FACS analysis was run on a BD FACSAria™ III cell sorter (BD Bioscience, flow cytometry facility, University of Zurich) using the following gating strategy. After the neutral density filter was removed from the machine, events were defined using an "SSC height AND FSC height" operator. The PBS as well as the BHI control was analyzed to establish the background noise, and the bacteria population was defined on an FSC-H vs SSC-H plot. Within the bacteria population, singlets were gated on an FSC-H vs FSC-A plot. Before sorting, GFP-positive/ negative gates were established by running 10'000 events of the FSL G3-0099 and the FSL G3-0007 controls. The flow rate was set as high as possible without losing efficiency. To sort activated FSL G3-0073 reporter cells into "PrfA active" and "PrfA non-active" cells the following protocol was used: the top 10% PrfA activating and non-activating respectively, were selected on a GFP-H histogram and sorted into two different

tubes. Flow cytometry data were analyzed using the FlowJo-Software for Windows (Version 10.0.8). The analysis was started by defining the cell signals, that counted as bacterial population. In a next step the cell population was further narrowed down by gating the single cells only. After those basic settings, the GFP+ and – cell populations were defined by using our positive (FSL G3-0099) and negative control (FSL G3-0007) as an objective standard. In order to compare fluorescence intensity across samples, the mean fluorescent intensity (MFI) of each sample was calculated using FlowJo.

CaCo-2 cell invasion assay

The human epithelial colorectal adenocarcinoma cell line, CaCo-2 (passages 40-56), was used for a gentamicin exclusion assay (Braun et al. 1998). Cells were grown in Minimal Essential Medium (MEM) (Ref no. 42360-024, Gibco, Switzerland) complemented with 20% fetal bovine serum (FBS) (Ref no.10270106, Thermo Fisher, Switzerland) and incubated at 37°C, 5% CO_2. 14-16h before the assay, 96-well plates (TPP AG, Trasadingen, Switzerland) were seeded with 10^4 CaCo-2 cells per well and grown to confluence for 24h. 15 minutes prior to the infection, cells were washed with PBS (PBS tablets, Gibco, Switzerland) once and the medium was changed to MEM without FBS. Sorted populations of *L. monocytogenes* FSL G3-0073 containing (i) cells with high PrfA acitvitiy and (ii) cells with low PrfA activity were used to infect CaCo-2 cells. Obtaining enough sorted bacteria of to enable an adequate multiplicity of infection (MOI) was a tradeoff between the time needed to sort and the necessity not to protract the experiment's time frame. 100ul of the sorted bacteria were added to the CaCo-2 cells in the 96 well plate to obtain a multiplicity of infection (MOI) of at least 10. As an internal control, 10ul of log phase cultures of the strains FSL 10403s (fully invasive) and *L. innocua* JF 5051 (non-invasive) were used, resulting in a similar MOI. One well was left out during infection as a sterility control (no bacteria control). Another cell-free well was loaded with 10ul of 10403s log phase culture to test whether the plate material itself

influences the invasion assay readout by binding bacteria (no cells control). After inoculation, the bacteria were brought in close contact with the host cells, by short-spinning the plate at 1000xg for 30s immediately after the infection (t=0). After an incubation time of 30min, non-adherent bacteria were removed by washing the cells once with sterile PBS. To kill extracellular bacteria, the Caco-2 monolayers were then overlaid with DMEM plus 100 µg/ml gentamicin (Gibco) and incubated for another 45min. At t= 90min post infection, each well was washed five times using sterile PBS. Host cells were lysed by adding 250µl PBS containing 40mg/ml saponin (Riedel-de Haën, Seelze, DE) to recover intracellular bacteria. Due to the fragility of the cell layer, all liquids were removed by using a multichannel pipet instead of a suction device and the washing steps were performed by gently adding the liquid at the wall of the well. To determine recovery rates, viable bacteria were quantified by spread-plating appropriate dilutions on to BHI agar plates. All experiments were repeated at least three times.

PrfA+/- passages

FSL G3-0073 *L. monocytogenes* cells were PrfA activated and sorted as described above. The sorted populations were inoculated into BHI broth and grown overnight at 37°C. Glycerol stocks were prepared the following day and designated p1+ for the obtained passage 1 PrfA+ and p1- for passage 1 PrfA-. For the next passage, BHI broth was inoculated directly from frozen stock to keep the generation number between sorts low. The next passage was established by repeating the above protocol until 6 passages (p6+ and p6-) were obtained.

All six passages were then analyzed in parallel by growing log phase cultures and activating PrfA as described above. After activation, the bacteria were fixed with 1% paraformaldehyde (PFA) and analyzed for GFP expression at the FACS. 10403s and FSL G3-0099 were included as negative and positive GFP controls, and FSL G3-0073 was included to establish the baseline fluorescence at passage 0.

Hemolysis assay

To obtain pure erythrocytes, full blood (human blood provided in EDTA tubes from Blutspende Zürich, CH) was centrifuged, the plasma supernatant removed and the erythrocytes were washed twice in equal amounts of NaCl and finally resuspended and diluted to 2% in PBS. To guarantee the same conditions, we used fresh blood (collected only one day before the experiment) with the same blood group (A+) for each experiment.

To analyse the hemolytic activity of the LLO secreted by *L. monocytogenes,* log phase cultures of the samples (p6+, p6-), as well as three controls (FSL G3-0073, B2-0046, B2-0237), were grown as described above. 2ml log phase culture was centrifuged (5min at 4000rpm/ 2397g), resuspended in equal amounts of PBS pre-warmed to either 37°C or 45°C and incubated at either 45°C for 15min, followed by 105min at 37°C or at 37°C for 120min. After this PrfA induction step, the cultures were centrifuged at 2400 x g for 5min, the cell-free supernatants were reduced by adding 5mM dithiothreitol (DTT) and incubated at 37°C for 1 h. Equal amounts of reduced supernatant and 2% diluted erythrocytes (either bovine or human) were mixed and incubated for 40min at 37°C in 96-well plates (Schärer, Stephan, and Tasara 2013; Vadia et al. 2011; Evans et al. 2013). The absorbance at 420 nm was measured using a Synergy HT plate reader (Biotek, Luzern, CH) and data were analyzed using the Gen5 software (Version 2.01 Biotek). PBS and 0.1% Triton X-100 instead of culture supernatant served as chemical controls (negative and positive, respectively). As biological controls, we included B2-0046 (*ΔprfA*) as negative and B2-0237 (PrfA*) as positive control in the assay. Hemolytic activities are expressed relative to the hemolysis of the FSL G3-0073, which was set to 100%.

Genome sequencing and analysis

To screen for mutations or differences in methylation patterns between the parent FSL G3-0073 strain and the derivative passage 6 (ILS G3-0005, ILS G3-0006) cultures, we sequenced

these strains by PacBio sequencing. To obtain a clonal population for the sequencing, a BHI plate was streaked out for ILS G3-0005 and ILS G3-0006 respectively. Subsequently, six colonies each were picked and PrfA activation induced as described above. To find the most extreme PrfA phenotypes, the activated and fixed *Listeria* were analyzed using a BD LSRFortessa™ cell analyzer.

The median GFP-H from three replicates was analyzed and presented relative to the positive control (Mean relative GFP-H) (table 2). For sequencing, a colony was chosen according to the following selection criteria: (i) high/low relative GFP-H and (ii) less than 5% overlap comparing the GFP-H of the positive colonies with the GFP-H of their according negative colonies.

DNA extraction was performed using the Sigma's GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, Switzerland) according to the user protocol. The single-molecule real-time sequencing was performed on a Pacific Biosciences RSII device at the Functional Genomics Centre Zurich (FGCZ). Library prep was conducted using PacBio Big Library Reagents / PacBio Blue Pippin Reagents, and three SMRT cells were used per strain. Reads were either (i) assembled against the *L. monocytogenes* 10403s reference sequence using the "Resequencing 1" pipeline or (ii) de novo assembled using the "HGAP assembly 2" pipeline within the SMRT Analysis 2.3.0 software (PacBio, Menlo Park, USA). SNP calling was by aligning the de novo assembly of the FSL G3-0073 and ILS G3-0005 sequencing results the 10403s reference sequence in Geneious (Version 9.0.5, Biomatters Inc.) using their proprietary assembler and variant calling algorithm.

Base modifications were determined using the "RS motification and motif analysis" pipeline within the SMRT Analysis 2.3.0 software (PacBio, Menlo Park, USA). The methylation patterns and motifs were then analyzed using CLC workbench 7.7.3 (Qiagen, Aarhus).

Statistical analysis

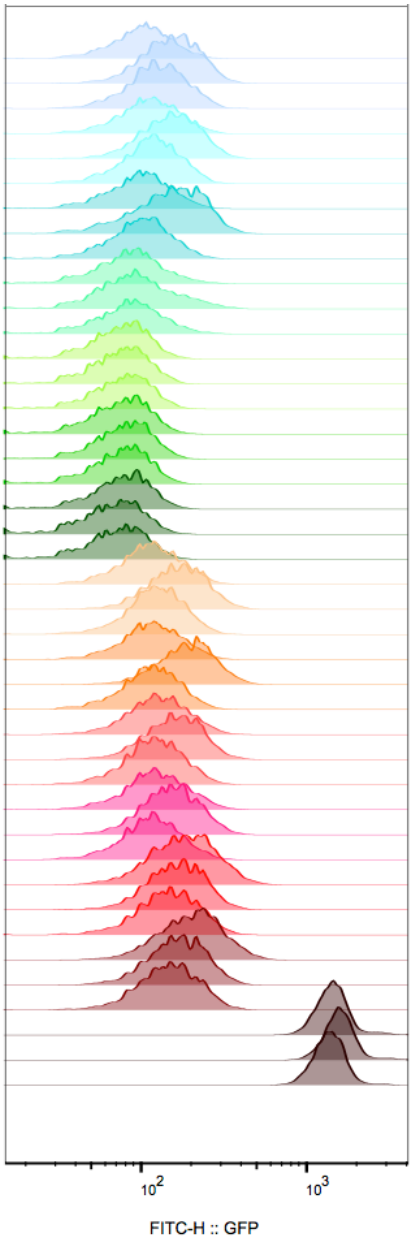
All experiments, except sequencing, were performed independently at least three times and analyzed in triplicate for each experiment. All statistical analyses were performed in R version 3.2.1 (R Core Team 2015), using ggplot2 version 2.1.0 for visualization (Wickham 2009).

To analyze the cell invasion assays, for both the PrfA+ and PrfA- samples, a Chi-square distribution of 10000 replicates was calculated based on the observed colony count and the dilution factor. The Chi Square distribution was based on the Bayes-Jeffrey prior for a Poisson distribution which defines as the upper and lower interval constructed from the Chi - Square distribution with degrees of freedom 2x+1 where x is the observed count (Maxwell, 2011). From these, a probability distribution for the fraction of recovered cells was calculated. Samples were considered significantly different in invasion efficiency if there was less than 5% overlap between the resulting probability distributions (corresponding to $p < 0.05$).

Results

A stable fluorescent phenotype occurs in sorted PrfA+ and - cells over six passages.

The FACS analysis of all 6 PrfA positive passages displayed a constant increase in fluorescence, while all six PrfA negative passages displayed a decrease in fluorescence (Median GFP-H) (Figure 1).



	SampleID	Subset Name	Count	Median : FITC-H	Date
	passage 1 PrfA-	Single Cells	9150	102	May 23rd
	passage 1 PrfA-	Single Cells	9209	153	May 27th
	passage 1 PrfA-	Single Cells	9049	125	June 7th
	passage 2 PrfA-	Single Cells	9231	112	May 23rd
	passage 2 PrfA-	Single Cells	9263	152	May 27th
	passage 2 PrfA-	Single Cells	8999	116	June 7th
	passage 3 PrfA-	Single Cells	9219	99.0	May 23rd
	passage 3 PrfA-	Single Cells	9292	152	May 27th
	passage 3 PrfA-	Single Cells	8948	100.0	June 7th
	passage 4 PrfA-	Single Cells	9204	85.9	May 23rd
	passage 4 PrfA-	Single Cells	9178	91.0	May 27th
	passage 4 PrfA-	Single Cells	9025	85.9	June 7th
	passage 5 Prfa-	Single Cells	9202	77.0	May 23rd
	passage 5 PrfA-	Single Cells	9182	77.0	May 27th
	passage 5 PrfA-	Single Cells	8977	79.0	June 7th
	passage 6 PrfA-	Single Cells	9049	78.0	May 23rd
	passage 6 PrfA-	Single Cells	9227	81.0	May 27th
	passage 6 PrfA-	Single Cells	8863	77.0	June 7th
	G3-0007	Single Cells	9152	78.0	May 23rd
	G3-0007	Single Cells	8996	69.0	May 27th
	G3-0007	Single Cells	9074	73.0	June 7th
	passage 1 PrfA+	Single Cells	9227	114	May 23rd
	passage 1 PrfA+	Single Cells	9215	167	May 27th
	passage 1 PrfA+	Single Cells	7582	123	June 7th
	passage 2 PrfA+	Single Cells	9303	116	May 23rd
	passage 2 PrfA+	Single Cells	9286	191	May 27th
	passage 2 PrfA+	Single Cells	9021	115	June 7th
	passage 3 PrfA+	Single Cells	9182	126	May 23rd
	passage 3 PrfA+	Single Cells	9234	158	May 27th
	passage 3 PrfA+	Single Cells	8952	120	June 7th
	passage 4 PrfA+	Single Cells	9153	122	May 23rd
	passage 4 PrfA+	Single Cells	9177	150	May 27th
	passage 4 PrfA+	Single Cells	8949	116	June 7th
	passage 5 PrfA+	Single Cells	9171	182	May 23rd
	passage 5 PrfA+	Single Cells	9152	164	May 27th
	passage 5 PrfA+	Single Cells	8896	146	June 7th
	passage 6 PrfA+	Single Cells	9003	203	May 23rd
	passage 6 PrfA+	Single Cells	9102	166	May 27th
	passage 6 PrfA+	Single Cells	8754	148	June 7th
	G3-0099	Single Cells	9085	1415	May 23rd
	G3-0099	Single Cells	8991	1561	May 27th
	G3-0099	Single Cells	9098	1356	June 7th

Figure 1 Fluorescence trend in PrfA+ and PrfA- cells over six passages: FACS analysis of all 12 passages (greenish PrfA- and reddish PrfA+) including the positive (FSL G3-0099) and negative control (FSL G3-0007). The range of analysed single cells was between 7582 and 9303. The fluorescence of each passage is shown as Median GFP-H. Data are based on three independent replicates on 3 different days (May 23rd, 27th and June 7th).

We observed no further change in fluorescence from passage 5 onwards and therefore stopped passaging after the sixth sorting and decided to further analyze these cells. Comparing passage six strains, the relative mean fluorescent intensity (MFI) was significantly higher in p6+ than in p6- ($p<<0.05$) (Figure 2), indicating stable PrfA phenotypes in passage six.

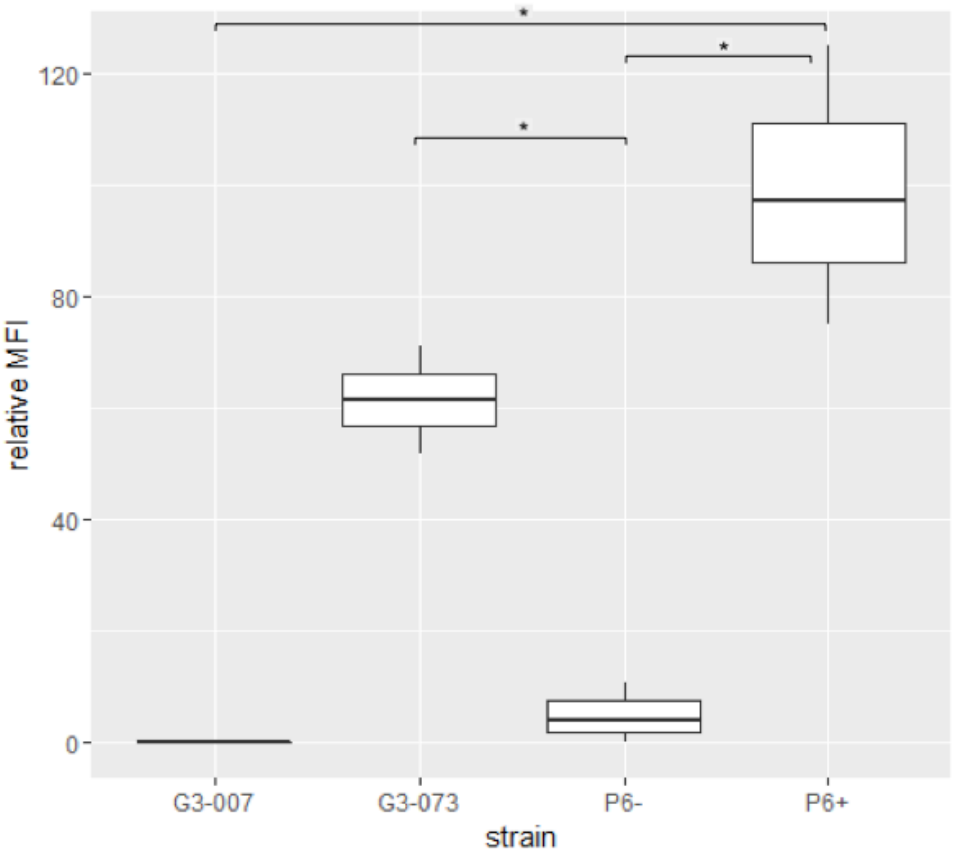
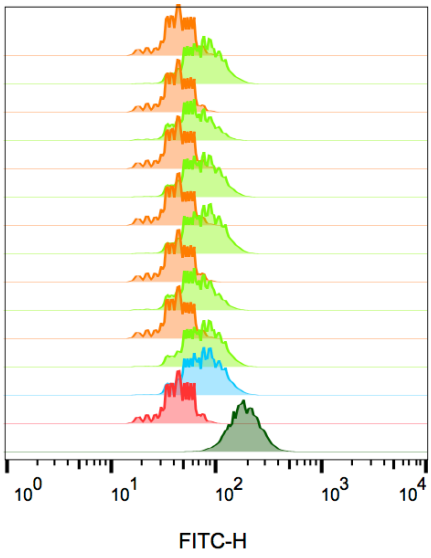


Figure 2 Mean relative fluorescent intensity of FSL G3-0007, FSL G3-0073, Passage 6+ and 6-: Shown is a boxplot of the MFI of p6+ and p6-, as well as the parent strain FSL G3-0073 and the negative control FSL G3-0007, measured on three independent days. p6+ differs significantly from p6- ($p<<0.05$) as well as p6+ from the negative control and p6- from the parent strain.

To further characterize the passage six strains, we (i) selected two individual colonies of p6+ and p6- for sequencing, (ii) sequenced the genomes using PacBio sequencing and (iii) performed phenotypic assays to determine if the observed strong PrfA activation pattern in p6+ had an effect on hemolytic activity or cell invasion efficiency.

We analyzed six individual p6+ and p6- colonies for their GFP expression after exposure to 45°C in PBS to induce PrfA activity. Throughout all three replicates the six PrfA- colonies

showed astonishingly low levels of GFP comparable to those of the negative controls. The mean relative MFI values for the six PrfA- colonies over all three replicates resulted in 19% and varied less than 1 % within one day. The mean relative MFI values for the six PrfA+ colonies over all three replicates was 39% and varied up to 8% within one day. The samples with the most extreme PrfA+/- phenotypes that were statistically different from each other in all three replicates (p6+ 6= ILS G3-0005; p6- 6 = ILS G3-0006) were selected for sequencing (Figure 3).



	SampleID	Subset Name	Count	Median : FITC-H
	p6- 6	Single Cells	74335	43.0
	p6+ 6	Single Cells	75181	76.0
	p6- 5	Single Cells	79626	43.0
	p6+ 5	Single Cells	76518	61.9
	p6- 4	Single Cells	74046	43.0
	p6+ 4	Single Cells	75744	74.0
	p6- 3	Single Cells	74492	43.0
	p6+ 3	Single Cells	74155	78.1
	p6- 2	Single Cells	74773	43.0
	p6+ 2	Single Cells	75576	63.1
	p6- 1	Single Cells	73952	43.0
	p6+ 1	Single Cells	75073	78.1
	G3-0073	Single Cells	75061	78.1
	G3-0007	Single Cells	110016	43.0
	G3-0099	Single Cells	99680	187

Figure 3 Analysis of individual passage 6 colonies: Depicted is one representative analysis out of 3 replicates. Specifically, six colonies of each FSL G3-0073 passage 6 positive and negative were picked, designated as p6+ 1-6/p6- 1-6 and analysed on three independent days at the FACS. 10403s as negative and FSL G3-0099 as positive control are pictured in red and dark green, respectively. The parent strain FSL G3-0073 is shown in light blue.

Table 2: The table shows the Mean relative GFP-H of all three replicates. On the basis of these data, the colonies for sequencing were picked.

Mean relative GFP-H	
p6+ 1	37.29
p6+ 2	32.80
p6+ 3	42.04
p6+ 4	41.63
p6+ 5	38.84
p6+ 6	42.92
p6- 1	19.35
p6- 2	19.25
p6- 3	19.25
p6- 4	19.25
p6- 5	19.12
p6- 6	19.25

p6+ strains show no increased hemolysis of human erythrocytes or increased invasion in CaCo-2 cells.

From the previous findings, we hypothesized that p6 cells would differ in PrfA-dependent phenotypes like hemolysis or host cell invasion. Criteria to choose an appropriate phenotypic assay were on the one hand a good visualization of the results and on the other hand an assay measuring the activity of one of the mainly PrfA dependent genes. *hly*, which codes for Listeriolysin O (LLO) seemed more than adequate considering that the reporter fusion was based on the *hly* promoter. Furthermore, the hemolytic ability of LLO provides a convenient readout. Therefore, we have decided to perform a hemolysis assays, which is widely used to detect LLO activity of *Listeria monocytogenes* strains (Schärer et al. 2013).

However, hemolysis assays with p6+, p6-, PrfA* and FSL G3-0073 showed no significant differences between p6+ and the parent strain (as determined by an explorative analysis of the data, Figure 4). In contrast, the controls that were included in the assay showed 183-fold higher hemolysis in the chemically lysed cells vs a NaCl control, and significantly different hemolysis levels of ΔPrfA and PrfA* control strains in all three replicates.

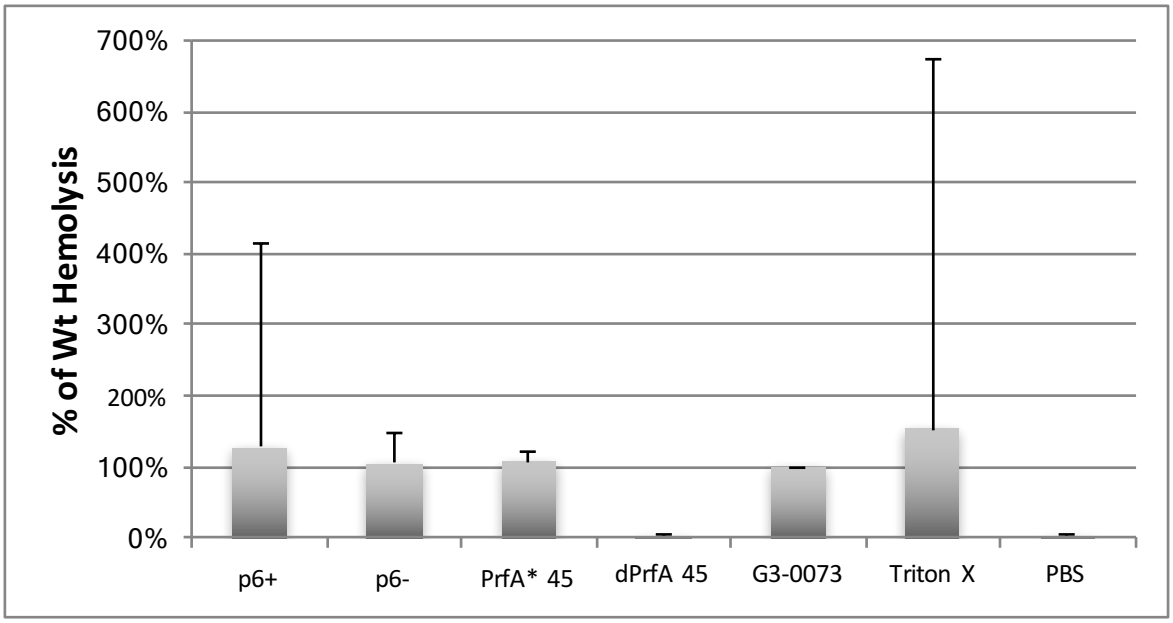


Figure 4 Hemolysis analysis in p6+/-: Hemolysis was tested by adding supernatant to a 2% human blood solution. The results are expressed relative to the wildtype strain that was set as 100%. Error bars indicate the standard deviation of the mean. Shown are, besides the combined p6 samples, the chemical controls represented by Triton X-100 and PBS and the biological controls, namely ΔPrfA and PrfA*. There was no significant difference between the sample p6+ and p6-.

For the invasion assays, the human colon carcinoma cell line CaCo-2 was used, imitating the natural entry pathway of the listeria cells. We included the combined p6 strains as well as the two of the six individual p6 colonies that showed the most extreme GFP phenotype in the ongoing analysis up to this point (ILS G3-0007 and ILS G3-0008). Both p6+ and p6- showed higher fractions of recovered cells than the parent 10403s wt strain ($p = 0.0096$ for p6+ vs 10403s parent) with no significant difference between the invasion efficiency of p6+ versus p6- (Figure 5).

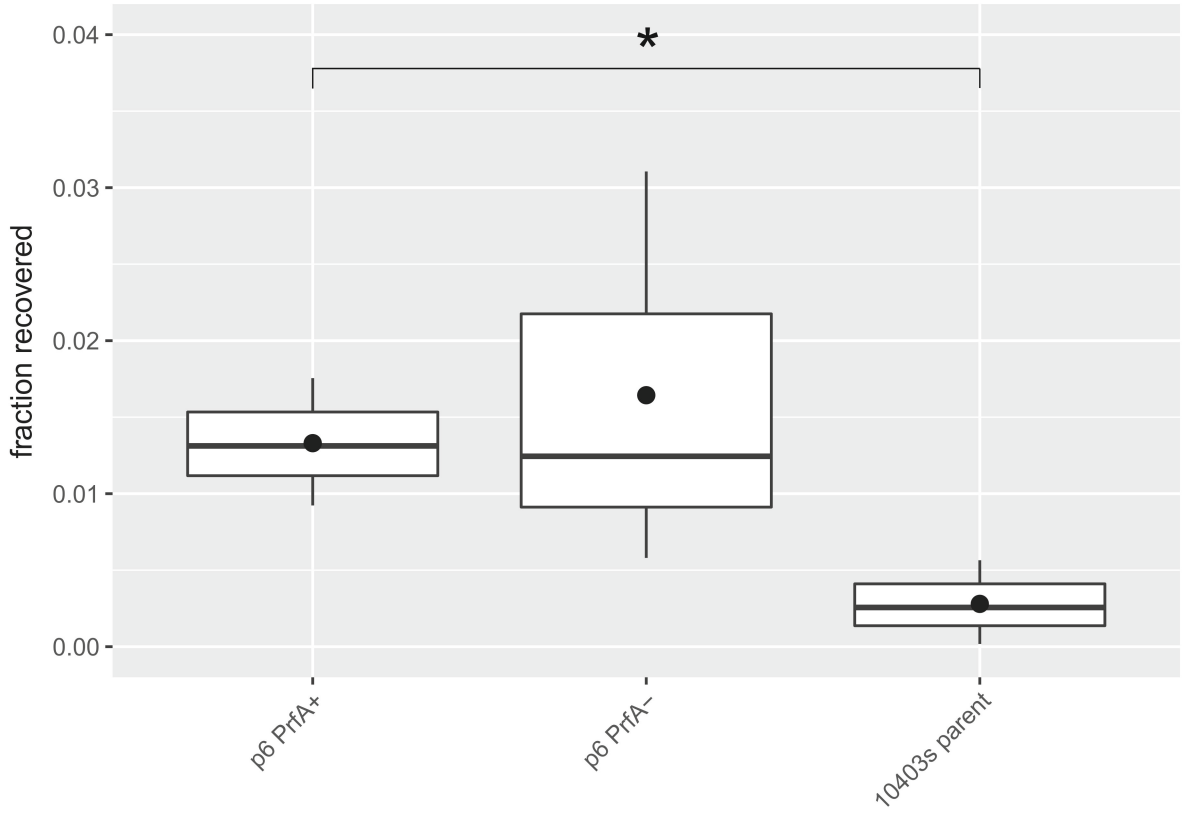


Figure 5 Invasion capacity of the combined p6 strains in Caco-2 cells: Shown is the fraction recovered of the samples p6+ and p6- in comparison to 10403s parent strain. There is no difference between the invasion ability of p6+ and p6-, however p6+ differs significantly from 10403s.

The system's sensitivity to differentiate between invasive and non-invasive bacteria was confirmed by the wildtype *L. monocytogenes* strain (10403s) and the non-invasive *L. innocua* strain (JF5051) which showed significant difference ($p<0.001$) in their invasion ability. As expected, the 'process control', showed similar invasion levels compared to the wildtype strain ($p>0.05$), indicating that the presence of the reporter construct did not negatively affect the

invasion efficiency. We observed no significant difference displayed the comparison of the 'no cells control' and the *Listeria innocua* strain ($p>0.05$), further proofing the soundness of the system.

PrfA+ and PrfA- induced and sorted *Listeria* cells of parent strain 10403s show no difference in host cell invasiveness

To test whether passaging negatively affected the invasiveness of the strains, we examined the invasion capacity of the induced and FACS-sorted *Listeria* cells of strain FSL G3-0073 by using a CaCo-2 infection model. The sorted PrfA+ and PrfA- populations did not significantly differ from one another in their invasion levels ($p>0.05$) (Figure 6).

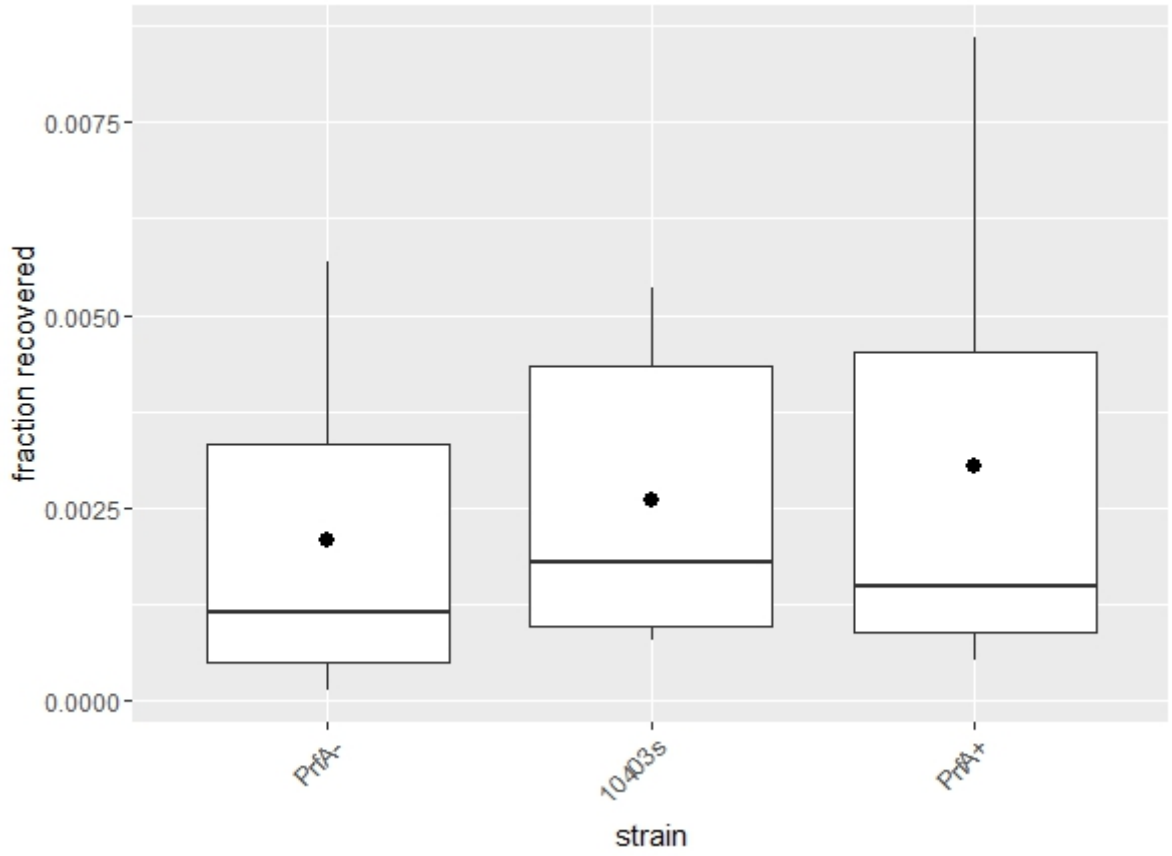


Figure 6 Invasion capacity of the induced and FACS-sorted Listeria cells in Caco-2 cells: Depicted is the fraction recovered of the PrfA+ and PrfA- population compared to 10403s parent strain. There was no evidence that PrfA+ and PrfA- differed statistically.

Genome sequencing and analysis

PacBio sequencing of all three strains resulted in a coverage of at least 1700x, a mean read length of 17000-19000bp and a post-filter quality score of 0.83. The assembly of the reads resulted in closed genomes for all three sequenced strains with the following sequence lengths for (i) the de novo and (ii) the 10403s reference assembly: (i) 2'910'118 bp and (ii) 2'903'122 bp for FSL G3-0073, (i) 2'910'127 bp and (ii) 2'903'119 bp for the ILS G3-0005 and in (i) 2'909'841 bp and (ii) 2'903'047 bp for ILS G3-0006. Given the massive coverage and the long read lengths, we decided to use the de-novo assemblies as an unbiased sequence for all further analysis. An initial screen showed that all three strains carried the wt PrfA sequence. The reporter construct at the pPL2 integration site was present in FSL G3-0073 and in ILS G3-0005, but lost from ILS G3-0006. The absence of the PrfA reporter construct from ILS G3-0006 meant that we had lost the ability to determine the status of PrfA activity in this strain and explained the consistently low levels of GFP fluorescence in the P6- strains. Therefore, the further analysis included FSL G3-0073 and ILS G3-0005 only.

For SNP calling, the de novo assembled sequences of FSL G3-0073 and ILS G3-0005 were aligned, revealing 172 variants in ILS G3-0005. Among those, two variants seemed interesting in the context of PrfA activation: several SNPs and an insertion in the coding sequence of *LMRG_02823*, and a C>A substitution within the 5'UTR of the internalin *LMRG_00195* (Figure 7). The remaining SNPs were either located within homopolymeric tracts which we deemed of low relevance due to the frequency at which sequencing mistakes occur within homopolymeric tracts, or silent mutations in coding sequences.

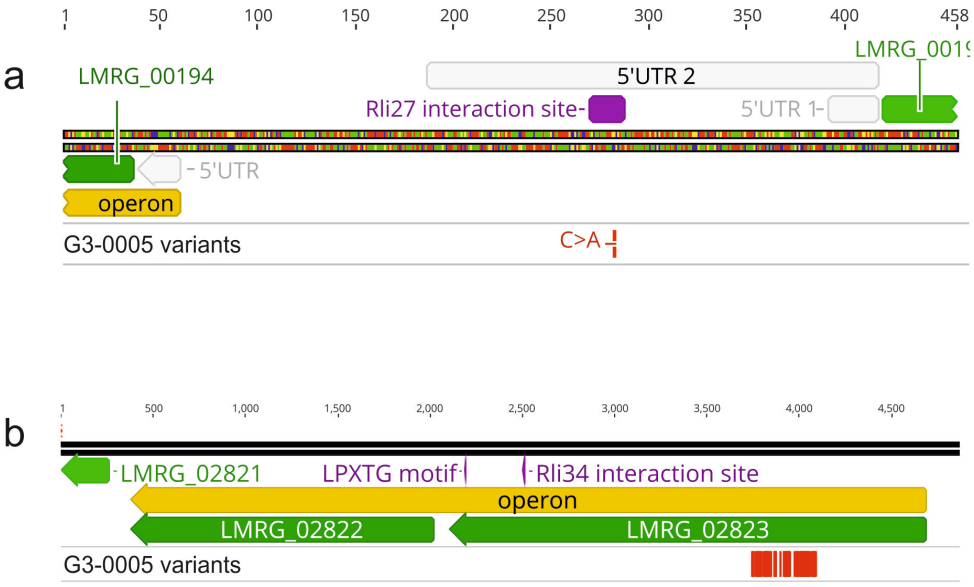


Figure 7: Dipicted are the loci of the two relevant variants found in ILS G3-0005 compared to the FSL G3-0073 parent strain. a) C>A substitution within the 5'UTR of the internalin *LMRG_00195* b) several SNPs and an insertion in the coding sequence of *LMRG_02823*

Discussion

The aim of this study was to analyze mechanisms behind PrfA activation at the single cell level in *L. monocytogenes*. Furthermore, we investigated the heritability of this activation property.

Our results support a hereditary component in PrfA activation at the single cell level, which we further analyzed to identify genetic as well as epigenetic mechanisms behind those observations by PacBio Sequencing.

Knowing that conformational changes lead to a constitutively active PrfA* mutation with constant high levels of active PrfA (Ripio et al. 1997; Vega et al. 2004), we had speculated that the reason for a stable PrfA+ phenotype we observed in ILS G3-0005 was a PrfA* mutation. However, the presence of the wt *prfA* sequence in both FSL G3-0073 and ILS G3-0005 suggested a heritable mechanism for the *prfA* activation other than PrfA*. Interestingly, we found two mutations with a possible effect on the function of LPXTG-family surface proteins.

Among the SNP's located within coding sequences, LMRG_02823 stands out in that it was the only one that had accumulated multiple SNP's and an 18bp insertion in ILS G3-0005 vs FSL G3-0073. LMRG_02823 is a putative peptidoglycan bound protein with an LPXTG motif. LMRG_02823 carries a series of tandem repeats that are part of the original as well as the extended multilocus variable number tandem repeat analysis (MLVA) scheme (Sperry et al. 2008; Chenal-Francisque et al. 2013). Toledo-Arana et al. (2009) found an upregulation of the LMRG_02823 homolog lmo1799 in the intestinal phase of infection and in human blood. Lmo1799 is transcribed in an operon together with lmo1798 (which corresponds to LMRG_02822), and the small RNA Rli34 interacts with the lmo1799 mRNA (Toledo-Arana et al. 2009). We hypothesize that the 18 bp insertion we found near the N-terminus of LMRG_0823 possibly affects protein function, while the SNP's are silent mutations within

the repeat region and may be due to sequencing errors. Given that the insertion is in frame, we exclude a polar effect on LMRG_02822, which codes for a hypothethical glucosyltransferrase. The insertion is outside of the LPXTG motif, the Rli34 interaction site or the repeat region and does not contain the CATCGG repeat sequence identified by Sperry et al. (2008). The functional effect of the observed insertion on protein function will be the subject of further analysis.

The mutation in the 5'UTR of *LMRG_00195,* an LPXTG family internalin, may affect the post-transcriptional regulation. Its EGDe homolog Lmo0514 is upregulated during growth of *L. monocytogenes* in human blood or the murine intestine (Toledo-Arana et al. 2009), in intracellular bacteria isolated from cell cultures (García-del Portillo et al. 2011; Quereda et al. 2014), and it is involved in the acid stress response (Quereda et al. 2016). A Δ*lmo0514* mutant was slightly attenuated *in vivo* (Quereda et al. 2016). Loh et al. (2006) predicted three transcriptional start sites *in silico*, of which the two predicted SigA-dependent promoters have been experimentally confirmed by Quereda et al. (2016). Specifically, they found an increase of the longer of the two Lmo0514 transcripts inside host cells, which was only efficiently translated in the presence of the small RNA Rli27 (Quereda et al. 2014). In accordance with this, Rli27 was also found to be upregulated in blood and in mice (Toledo-Arana et al. 2009). Rli27 interacts with the 5'UTR of *lmo0514* via two core regions, possibly resulting in the unmasking of the ribosomal binding site on the *lmo0514* mRNA (Quereda et al. 2016). ILS G3-0005 carries a C > A substitution at position -137 of *LMRG_00195*. Interestingly, this mutation is located within a three nucleotides region that has been shown to abolish Rli27 binding to the *lmo0514* 5'UTR when mutated (corresponding to the "Rli27 mut1" variant analyzed by (Quereda et al. 2014). It is conceivable that the interruption of the binding of Rli27 to the *LMRG_00195* 5'UTR leads to a broken feeback loop with a constant signal resulting from the low internalin levels on the cell surface. However, a direct link to PrfA is

not immediately obvious since neither *lmo0514* nor Rli27 were found to be regulated by PrfA (Quereda et al. 2016).

Alternatively, it is possible that the two mutations in membrane proteins that we found could be due to heat stress and have nothing to do with PrfA activity. This could be further investigated by determining whether the 45°C heat stress used in the experiments performed here also leads to differential expression of *LMRG_00195* and LMRG_02823 and whether mutants have growth defects under heat stress.

The stably high PrfA activity in ILS G3-0005 upon PrfA induction with heat stress would suggest that this strain expresses other, PrfA-dependent phenotypes which would support our observations.

While performing the phenotypic assays, we did not yet know about the sequencing results of ILS G3-0005 and ILS G3-0006, therefore our analysis was based on the hypothesis that the hemolytic activity of p6+ to be higher than of p6-. With the updated knowledge about p6- lacking the reporter fusion, the hypothesis was reformulated and the data reanalyzed, now comparing p6+ with the WT strain. Our new hypothesis was: the hemolytic activity of p6+ is higher than of the parent strain.

Because of the high variance between the three replicates of the hemolysis assay, there was no statistical evidence of differences in hemolytic activity between p6+, p6-, PrfA* and FSL G3-0073 (Figure 4), suggesting that there is no difference in hemolytic activity or, alternatively, that the assay was not sensitive enough to detect subtle differences in hemolytic activity. In fact, it seemed that strains with any hemolytic property triggered strong hemolysis, independent of the LLO quantity. The included controls suggested that the assays are capable of determining presence/absence of LLO; the ΔPrfA and PrfA* control strains showed significantly different hemolysis levels in all three replicates and we detected a 183-fold higher hemolysis in chemically lysed erythrocytes vs the NaCl negative control.

Because PrfA positively regulates all virulence factors needed for the invasion of the host cell (Freitag et al. 2009), the next phenotypic approach was to perform CaCo-2 cell invasion assays with the following hypothesis: the cell invasion ability of p6+ is better than of p6-. Again, realizing that the reporter construct was lost from ILS G3-0006, the hypothesis was adapted to: the cell invasion ability of p6+ is better than of the wildtype strain. Comparing the invasion capacity of ILS G3-0007 (p6+) and the wt strain, we found that they are significantly different from each other ($p = 0.0096$). However, 10403s was included in the assays as a procedural control to ensure that each assay differentiated between the invasive *L. monocytogenes* and the non-invasive *L. innocua* strains. Any experiment that did not fulfill these basic criteria would have been excluded from the analysis. Given this role in the experimental setup, neither *L. monocytoenes* 10403s nor *L. innocua* JF5051 were heat activated at 45°C like the passages. The difference in invasion efficiency between p6+ and 10403s can either be due to inherently higher PrfA activity in p6+, or to the different treatment of the cells prior to the invasion assay. This second scenario would lend further support to the somewhat unexpected finding by Guldimann et al. (submitted) that exposure to 45°C heat leads to strong activation of PrfA in *L. monocytogenes*. A comparison of p6+ and the 10403s strain under the same conditions, namely the activation of PrfA, could clarify this issue.

In conclusion, our results support the hypothesis that the PrfA activation pattern is an inherent property of the cell that is hereditary over at least six passages. The fact that two LPXTG-family surface proteins, both regulated by small RNA's, are associated with the high-PrfA-activating strain is noteworthy and will be subject to further analysis.

The hemolysis assays and the CaCo-2 invasion experiments included in this work could not conclusively establish a PrfA-dependent phenotype for the strongly PrfA activating strain G3-0005.

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